REMARKS

I. Detailed Action

The Examiner objects to claims 88 and 91 for informalities. Applicants respectfully submit that claims 88 and 91 have now been amended and are in proper condition for allowance. Reconsideration is requested.

Applicants acknowledge after careful reading of the Specification that several minor spelling and punctuation mistakes were found. The specification has now been amended to correct these minor changes that do not add new matter.

II. Claim Rejections – 35 U.S.C. § 112, First Paragraph

Claims 83-85 and 98 stand rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. The Examiner states that the specification is enabling for "how to make transgenic tobacco and tomato plants that express recombinant hepatitis B viral surface antigen proteins". However, the Examiner states that the specification does not disclose how to "make transgenic plants that express a recombinant animal viral antigen protein at a level of about .03% or more of total soluble protein, at a level of about .05% or more of total soluble protein, or at a level of about .1% or more of total soluble protein". In addition, the Examiner states that Applicants have not disclosed the type of vector to be used therefore the "specification does not provide sufficient guidance for one of skill in the art to make transgenic plants that express a recombinant animal viral antigen protein at the claimed levels".

Applicants respectfully traverse this rejection. Attached herewith for the Examiner's consideration is the §132 Declaration of Dr. John Howard discussing Applicants ability to overcome the technical difficulties of expression of viral antigens in plants which subsequently will produce an immune response in a human or animal when consumed as food (page 14, specification). This Declaration was also submitted in the prosecution of the parent application of this invention (USSN 09/111,330). As exemplified in the Declaration, processing of heterologous protein products in plants may produce an altered structure, or lead to miscompartmentalization, or be processed in low yields (paragraph 5, Declaration).

Further, as exemplified in Fryxell, membrane bound proteins are difficult to express and typically achieve only very low expression levels (paragraph 6, Declaration). Such difficulties and low expression levels would not leave one of skill in the art to expect that an immune response could be generated when a transgenic plant producing antigen at this low level is introduced. However, Applicants have accomplished exactly what one skilled in the art thought could not be done. As described in paragraph 9 of the Declaration, Applicants conducted experiments which follow the teachings of the specification whereby high expression of viral antigens in plants was achieved (page 3, Declaration). This was the first known clear ability to express viral antigens at a detectable level in plants thereby eliciting an immune response.

Applicants also provide, with this amendment, a copy of Gomez et al., "Expression of Immunogenic Glycoprotein S Polypeptides from Transmissible Gastroenteritis Coronavirus in Transgenic Plants" Virology, 249:352-358 (1998). In this article, the authors cite inventor, Mason's 1992 description of the present invention as the first introduction of vaccine production in transgenic plants. (See p. 352, col. 2 citing Mason et al., "Expression of Hepatitis B Surface Antigen in Transgenic Plants", Proc. Natl. Acad. Sci., 89:11745-11749 (1992), copy also attached). They discuss the Mason descriptions and follow its teachings to expression TGEV in *Arabidopsis*. Thus, Gomez et al. recognizes the present invention as the first to actually teach viral expression in plants and uses Applicants teachings to later express the TGEV protein.

As also described in the Declaration, the Applicants are able to achieve expression levels of animal viral antigens using the processes described in the application which have not before been achieved. As the Declaration demonstrates, previous expression levels have been so low that an immune response is not expected. For the first time, however, the Applicants have expressed viral antigens at high levels in plants. As the data demonstrates, a viral protein, optimized for plants, was expressed in leaf of corn at up to 0.1% of total soluble protein. To date, no demonstration has been previously made of expression levels this high. Applicants do include claims which recite these levels of expression to further define the invention. Applicants respectfully submit that the specification does provide sufficient

guidance for one of skill in the art and that through this Declaration, it is clear that the specification is enabling.

In addition, the Examiner states that the Applicants have not disclosed the type of vector to be used. Applicants respectfully traverse this rejection. The specification teaches the various methods of introducing foreign genes into both monocotyledonous and dicotyledonous plants. "The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include the following approaches: 1) Agrobacterium-mediated gene transfer; 2) direct DNA uptake, including methods for direct uptake of DNA into protoplasts, DNA uptake induced by brief electric shock of plant cells, DNA injection into plant cells or tissues by particle bombardment, by use of the micropipette systems, or by the direct incubation of DNA with germinating pollen; or 3) the use of plant virus as gene vectors" (page 18, specification). Further, the application goes on to specifically detail the use of vectors through the various methods of directing DNA transfer into plant cells thereby teaching a plasmid vector for transforming a plant with a promoter operably-linked to a DNA sequence encoding a recombinant viral antigen that is antigenic to a human or an animal (page 20-28, specification).

The Examiner further states that the specification is not enabling in disclosing the "level at which a recombinant protein must be expressed in order for transgenic plant tissue to elicit an immune response against a viral antigen when said tissue is orally administered to an animal". In addition, the Examiner states that Applicants have not disclosed the "manner in which such tissue should be administered to an animal in order to elicit an immune response".

Applicants respectfully traverse this rejection. As stated above, the §132 Declaration submitted herewith follows the disclosure in the specification of the recombinant protein expression in transgenic plant tissue in order to elicit an immune response against a viral antigen protein which had not previously been achieved. Therefore, Applicants submit the present specification does provide for enablement. Further, the specification does teach the use of "oral vaccines in one or more tissues of a transgenic plant, thereby availing large human and animal populations of an inexpensive means of vaccine production and administration" (page 6, specification). The specification further teaches the oral vaccine

being produced in edible transgenic plants and then administered through the consumption of a part of those edible plants (page 8, specification). As described above, the transgenic plants will express a recombinant animal viral antigen protein at certain levels of expression of total soluble proteins. In addition, "the preparation of vaccines is generally well understood in the art (e.g., those derived from fermentative yeast cells known well in the art of vaccine manufacture) Valenzuela et al., *Nature* 298, 347-350 (1982), as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated by reference on page 16 of the specification. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared" (page 16, specification). The specification goes on to explain the conventional administration of vaccines thereby enabling one skilled in the art to orally administer such vaccines to animals.

Applicants assert that the amended and original claims 83-85 and 98 are enabled by the specification provided. Applicants therefore respectfully request reconsideration and the withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

III. Claim Rejections – 35 U.S.C. § 112, Second Paragraph

Claims 98-100 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner states that claim 98 is indefinite, as it is "unclear whether the plant or the plant tissue is expressing a recombinant protein". Claim 98 has now been amended to recite "wherein said plant tissue" thereby alleviating this rejection.

The Examiner further states that claims 99-100 are indefinite in their recitation of "said plant", as it is "unclear if the plant refers to a plant comprising a protein or a transgenic plant". Claims 99-100 have now been amended to specify "transgenic plant" thereby alleviating this rejection. Applicants assert all the claims are in a condition for allowance. Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

IV. Claim Rejections – 35 U.S.C. § 102(e)

Claims 73-75, 88, 91 and 99-100 stand rejected under 35 U.S.C. § 102(e) as being anticipated by Goodman et al. (U.S. Patent No. 4,956,282, September 11, 1990). The Examiner states that Goodman et al. teaches "transgenic plants expressing recombinant animal viral antigen proteins from leukemia and lymphotrophic retroviruses, herpes simplex virus, hepatitis B virus and adenovirus; transgenic plants expressing chimeric recombinant animal viral antigen proteins fused to a transit or leader peptide from a plant peptide; and the use of tomato plants to express these proteins as well as other edible plants". Therefore, the Examiner states "these plants would inherently possess the ability to elicit an antigenic response, and the viral proteins taught by Goodman et al. would inherently trigger the production of antibodies to a viral protein". The Examiner further notes that the reference does not teach "an antigenic response in animals upon oral administration of tissue obtained form the claimed plants".

Applicants respectfully traverse this rejection. The Goodman et al. reference simply provides a general disclosure of a means of expressing mammalian peptides in plant cells. Goodman et al. does not teach the use of viral antigenic proteins expressed in transgenic plants or the use of the proteins as a vaccine which elicit an immune response upon oral administration. Applicants respectfully submit that "for prior art to anticipate under 35 U.S.C. § 102, every element of the claimed invention must be identically disclosed, either expressly or under principles of inherency in a single reference." *Corning Glass Works v. Sumitomo Electric*, 9 U.S.P.Q.2d 1962, 1965 (Fed. Cir. 1989). The exclusion of a claimed element, no matter how insubstantial or obvious, from a prior art reference is enough to negate anticipation. *Id.* In order to be anticipated there must be a teaching of a viral, as opposed to bacterial or mammalian protein, properly folded (glycosylated) and finally that it expresses at adequate levels. *Ex parte Deuel*, 33 U.S.P.Q.2d 1445, 1451 (Bd. Pat. App. & Int'f 1993). This teaching cannot come from Applicants' own specification. *Id.*

In addition, the general rule of inherency is that it may be relied upon where and only where the consequences of following the references disclosure always inherently produces a result in the claimed invention. If there is not a reasonable certainty that the claimed subject

matter will necessarily result, the rejection fails. See, *W.L. Gore Associates, Inc. v. Garlock, Inc.*, 220 U.S.P.Q. 303, 314 (Fed. Cir. 1983). Instead, there must be a teaching or suggestion within the prior art to combine the particular elements as done by the inventor. Therefore, Applicants submit that Goodman et al. clearly does not anticipate claims 73-75, 88, 91 and 99-100 because the prior art does not teach (1) a transgenic plant expressing a recombinant viral antigen protein; (2) whereby this protein is antigenic to a human or an animal following administration of this protein either in an isolated purified form or as an ingestible transgenic plant or plant component. Nor can it hint at expression levels as recited in claims 83-85.

This reference can be clearly distinguished from the present application where a recombinant viral antigenic protein has been demonstrated to elicit an immune response when expressed in plant tissue and subsequently fed to animals to form antibodies. Goodman et al. provides a general disclosure of a means of expressing mammalian peptides in plant cells to achieve high yields which may be harvested (col. 1, line 41, lines 64-67). For example, the reference merely teaches the transformation of a dicotyledonous plant cell with a structural gene coding for an interferon whereby the interferon gene is expressed and harvested (col. 10, lines 39 and 50). The cited reference does not teach a specific means for expressing immunogen derived from a viral protein in a plant nor the administration of the claimed oral vaccine by consumption of the edible transgenic plant (page 7, specification). One skilled in the art would not expect membrane bound proteins as in the present invention, which are plagued with low expression levels, to be useful or determinable by Goodman et al. In fact, Goodman et al. at best contemplates the opposite, high production of recombinant mammalian proteins for harvesting only. Whereas the present application teaches expression of a viral protein which involves different mechanisms and concerns than expression of a mammalian protein in plants because a virus relies upon its host for proper expression and processing of the proteins produced. Goodman et al. does not discuss or teach how one might accomplish this result.

Further Goodman et al. does not teach an antigenic response in animals upon oral administration of tissue obtained from the claimed invention's transgenic plants. Goodman et al. only teaches the production of mammalian proteins and harvesting of such proteins for

later "integration of the construct into the plant genome under conditions where cells can be used to produce plants" (col. 2, lines4-6). In contrast, the present invention clearly teaches the expression of viral derived antigenic proteins in tomato plants, and the ability to elicit an immune response in an animal or human when ingested (page 24, specification). However, Goodman et al. merely teaches the transformation of tobacco with interferon whereby the interferon gene is expressed. Goodman et al. also does not teach the use of viral antigenic proteins expressed in transgenic plants or the use of the proteins as a vaccine to these same viruses or the administration of the vaccine to elicit an immune response.

As disclosed in the Declaration submitted herewith, the expression of heterologous DNA expressed in plants is unpredictable for several reasons, including incomplete transcription of the gene due to premature transcription termination, unexpected mRNA processing during transcription, inefficient or incorrect splicing or polyadenylation of mRNA, instability of cytoplasmic mRNA, inefficient translation of the cytoplasmic mRNA, or instability of the protein due to its acceptability to plant proteinases (paragraph 4, Declaration). Further, a plant's intracellular environment differs from the intracellular environment where the protein is normally expressed and may have a particular consequence of protein folding (paragraph 5, Declaration). This of course would have drastic effects on the ability of the protein to interact with antibodies to generate the appropriate immune response. As disclosed in the Declaration, Kenward et al. observed that for example, a fish antigen protein expressed in a plant was shown to be completely unstable at normal temperatures (paragraph 5, Declaration). Further, as described in the Declaration, processing of heterologous protein products in plants may produce altered structure, miscompartmentalization, or extremely low yields. For example, TGEV is a glycosylated protein and folding after protein production must match an active form of the protein in order to provide the appropriate antibody interaction (paragraph 6, Declaration). Hepatitis B, TGEV, and other membrane proteins also exhibit difficulties in expression. For example, at paragraph 6 of the Declaration, expression levels of various heterologous proteins in *Pichia pastoris* are discussed. It can be seen that membrane proteins typically achieve only very low expression levels, as much as 1,000 times lower expression than tumor necrosis factor or

Tetanus toxin. However, Applicants teach what one skilled in the art did not think was possible and that is to achieve high expression of viral antigens in plants which enables one ordinarily skilled in the art to then produce vaccines from edible transgenic plants thereby providing large human and animal populations an inexpensive and effective means of vaccine production and administration (pages6-7, specification).

The Examiner has not shown a teaching in the cited art that the vectors and/or methods used in expressing mammalian proteins in transgenic plants could be used successfully with Applicants' specific viral proteins. Moreover, it s well known in the art that while viruses rely on their host for proper processing, mammalian proteins are not only chemically different but also have their own processing mechanisms. The only means by which one of ordinary skill in the art could obtain the recombinant animal viral antigen protein, vaccines, and transgenic plants of claims 73-75, 88, 91 and 99-100 is through the use of impermissible hindsight reconstruction. At most, Goodman et al. is an invitation for one skilled in the art to attempt to express viruses in plants. However, it does not teach one skilled in the art the appropriate vectors, promoters, mediums, infection procedures, etc. to obtain viral immunogens derived from a hepatitis virus in a plant that are capable of producing a vaccine without an undue amount of experimentation. Thus, Goodman et al. does not provide sufficient description to one skilled in the art to practice the Applicants' invention.

In light of the above, Applicants submit that the present invention is clearly distinguished from and, therefore, not anticipated by Goodman et al. Applicants respectfully request reconsideration and withdrawal of the rejections to claims 73-75, 88, 91 and 99-100 under 35 U.S.C. § 102(e).

V. Double Patenting

Claims 73-75, 83-85, 88, 91 and 98-100 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 1 of U.S. Patent No. 5,612,487. Applicants are herein submitting a terminal disclaimer in compliance with 37 C.F.R. § 1.321(c), which disclaims any term of a patent issuing from this application which would extend beyond the term of U.S. Patent No. 5,612,487. Therefore, Applicants

submit that the claims are in proper form for allowance and respectfully request reconsideration and withdrawal of the obviousness-type double patenting rejection.

VI. Conclusion

In light of the above remarks, Applicants respectfully assert that claims 73-75, 83-85, 88, 91 and 98-100 are now in condition for allowance. Applicants respectfully request reconsideration and withdrawal of the above rejections.

A request for a one-month extension of time is being submitted in connection with this amendment; however, please consider this a request for any additional extension inadvertently omitted, and charge any additional fees to Deposit Account No. 26-0084.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "**Version with markings to show changes made.**"

Reconsideration and allowance is respectfully requested.

Respectfully submitted,

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Application No. 09/767,734

AMENDMENT — VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification

The paragraph at page 3, beginning at line 4, has been amended as follows:

Various avirulent viruses have been used as vectors. The gene for hepatitis B surface antigen (HBsAg) has been introduced into a gene non-essential for vaccinia replication. The resulting recombinant virus has elicited an immune response to the hepatitis B virus in test animals. Additionally, researchers have used attenuated bacterial cells for expressing hepatitis B antigen for oral immunization. Importantly, when whole cell attenuated *Salmonella* expressing recombinant hepatitis antigen were fed to mice, anti-viral T and B cell immune responses were observed. These responses were generated after a single oral immunization with the bacterial cells resulting in high-titers of the antibody. See, e.g., "Expression of hepatitis B virus antigens in attenuated *Salmonella* for oral immunization," F. Schodel and H. Will, *Res. Microbiol.*, 141:831-837 (1990). Others have had similar success with oral administration routes for recombinant hepatitis antigens. See, e.g., M.D. Lubeck et al., "Immunogenicity and [efficiacy]efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus," *Proc. Natl. Acad. Sci.* 86:6763-6767 (1989); S. Kuriyama, et al., "Enhancing effects of oral adjuvants on anti-HBs responses induced by hepatitis B vaccine," *Clin. Exp. Immunol.* 72:383-389 (1988).

The paragraph at page 5, beginning at line 1, has been amended as follows:

Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. Plants that contain the transgene in all cells can then be regenerated and can transfer the transgene to their offspring in a Mendelian fashion.[4] Both [monocotyledenous]monocotyledonous and [dicotyledenous]dicotyledonous plants have been stably transformed. For example, tobacco, potato and tomato plants are but a few of the [dicotyledenous]dicotyledonous plants which have been transformed by cloning a gene which encodes the expression of 5-enolpyruvyl-shikimate-3-phosphate synthase.[5]

The paragraph at page 17, beginning at line 30, has been amended as follows:

Since many edible plants used by humans for food or as components of animal feed are [dicotyledenous]dicotyledonous plants, it is preferred to employ dicotyledons in the present invention, although monocotyledon transformation is also applicable especially in the production of certain grains useful for animal feed.

The paragraph at page 18, beginning at line 18, has been amended as follows:

There are various methods of introducing foreign genes into both [monocotyledenous]monocotyledonous and [dicotyledenous]dicotyledonous plants.[33,34] The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include the following approaches: 1) Agrobacterium - mediated gene transfer;[35,36,37,53] 2) direct DNA uptake,[38] including methods for direct uptake of DNA into protoplasts,[8] DNA uptake induced by brief electric shock of plant cells,[41,42] DNA injection into plant cells or tissues by particle bombardment,[39,44-46] by the use of micropipette systems,[43,47,48] or by the direct incubation of DNA with germinating pollen;[40,49] or 3) the use of plant virus as gene vectors.[33,51]

The paragraph at page 18, beginning at line 26, has been amended as follows:

The Agrobacterium system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the Agrobacterium delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation.[6] The Agrobacterium system is especially viable in the creation of transgenic [dicotyledenous]dicotyledonous plants.

The paragraph at page 26, beginning at line 10, has been amended as follows:

A. tumefaciens was cultured in 50 milliliters (50 ml) of YEP (yeast extract-peptone broth)[58] containing two-tenths milligrams per milliliter (0.2 mg/ml) streptomycin until the optical density (O.D.) at 600 nanometers (nm) of the culture reaches about five tenths (0.5). The cells were then centrifuged at 2000 times gravity (2000XG) to obtain a bacterial cell pellet. The Agrobacterium pellet was resuspended in ten milliliters of ice cold one hundred fifty millimolar sodium chloride

(150mM $NaCl_2$). The cells were then centrifuged again at 2000XG and the resulting Agrobacterium pellet was resuspended in one milliliter (1 ml) of ice cold twenty millimolar calcium chloride (20mM $CaCl_2$). Five-tenths microgram (0.5 μg) of plasmid pHB101 or plasmid pHB102 was added to two tenths milliliters (0.2 ml) of the calcium chloride suspension of A. tumefaciens cells in a one and five tenths milliliter (1.5 ml) microcentrifuge tube and incubated on ice for sixty minutes. The plasmid pHB101 or pHB102 DNA and A. tumefaciens cells mixture was frozen in liquid nitrogen for one minute, thawed in a twenty-eight degree Celsius (28°C) water bath, and then mixed with five volumes or 1 milliliter (1 ml) of YEP (yeast extract-peptone broth). The plasmid pHB101 or pHB102 and A. tumefaciens mixture was then incubated at twenty-eight degrees Celsius (28°C) for four hours with gentle shaking. The mixture was plated on YEP (yeast extract-peptone broth) agar medium containing fifty micrograms per milliliter (50 μg/ml) kanamycin. Optimum drug concentration may differ depending upon the Agrobacterium strain in other experiments. The plates were incubated for three days at twenty-eight degrees Celsius (28°C) before selection of resultant colonies which contained the transformed Agrobacterium harboring the pHB101 or the pHB102 plasmids. These colonies were then transferred to five [millileters]milliliters (5 ml) of YEP (yeast extract-peptone broth) containing fifty micrograms per milliliter (50 μg/ml) of kanamycin for three days at twenty-eight degrees Celsius (28°C).

The paragraph at page 27, beginning at line 27, has been amended as follows:

The regenerated kanamycin-resistant pHB101 and pHB102 transformed tobacco plants were analyzed by hybridizing RNA samples with a ^{32}P [labelled]labeled probe encompassing the HBsAg gene coding region.

The paragraph at page 28, beginning at line 12, has been amended as follows:

Five micrograms of each RNA sample was denatured by incubation for fifteen minutes at sixty-five degrees Celsius (65°C) in twenty millimolar (20mM) MOPS (3-N-morpholino) propanesulfuric acid, pH 7.0; ten millimolar (10 mM) sodium acetate; one millimolar ethylenediaminetetraacetic acid (1 mM EDTA); six and one half percent (6.5% w/v) formaldehyde; fifty percent (50% v/v) formamide, and then fractionated by electrophoresis in one percent (1%) agarose gels. The nucleic acids were transferred to a nylon membrane by capillary blotting[59] for

sixteen hours in twenty-five millimolar (25 mM) sodium phosphate, pH 6.5. Then the nucleic acids were crosslinked to the membrane by irradiation with [utlraviolet]ultraviolet (UV) light and the membrane pretreated with hybridization buffer [twenty-five hundredths molar (.25M) sodium phosphate, pH 7.0; one millimolar ethylene diamine tetraacetic acid (1mM EDTA); seven percent (7%) sodium dodecyl sulfate (SDS)] for one hour at sixty-eight degrees Celsius (68°C). The membrane was probed with 10^6 counts per minute per milliliter (cpm/ml) ^{32}P-labelled random-primed DNA using a 700 base pair (bp) Bam HI-Acc I fragment from plasmid pKS-HBS which includes most of the coding region for HBsAg. Blots were hybridized at sixty-eight degrees Celsius (68°C) in hybridization buffer and washed twice for five hundred and fifteen minutes with forty millimolar (40 mM) sodium phosphate, pH 7.0 per one millimolar ethylene diaminetetraacetic acid (1mM EDTA) per five percent sodium dodecyl sulfate (5% SDS) at sixty-eight degrees Celsius (68°C) and exposed to X-OMAT AR film for twenty hours.

The paragraph at page 31, beginning at line 17, has been amended as follows:

Tomato, Lycopersicom esculentum var. VFN8, was transformed as in Example II. B and C by the leaf disc method using Agrobacterium tumefaciens strain LBA4404 as a vector, McCormick et al., 1986.[23] A. tumefaciens cells harboring plasmid pHB102, constructed as in Example II. A.2, which carries the HBsAg coding region fused to the tobacco etch virus untranslated leader, Carrington & Freed, 1990,[73] and the cauliflower mosaic virus 35S promoter, were used to infect cotyledon explants from seven day old seedlings. The explants were not preconditioned on feeder plates, but infected directly upon cutting, and co-cultivated in the absence of selection for two days. Explants were then transferred to medium B, McCormick et al., 1986,[23] containing five-tenths milligrams per [millilter]milliliter (0.5 mg/ml) carbenicillin and one-tenth milligram per milliliter (0.1 mg/ml) kanamycin for selection of transformed callus. Shoots were rooted in MS medium containing one-tenth milligram per milliliter (0.1 mg/ml) kanamycin but lacking hormones, and transplanted to soil and grown in a greenhouse.

The paragraph at page 31, beginning at line 29, has been amended as follows:

Several independent kanamycin-resistant callus lines were obtained after Agrobacterium-mediated transformation of the tomato variety VFN8. One of these lines regenerated shoots with

high [freguency]frequency and was rooted and grown in soil in the greenhouse. The tissues from these plants were used for the protein and RNA analyses.

In the Claims

Please amend claims 88, 91 and 98-100 as follows:

88. (Twice Amended)

The transgenic plant of claim 73, said plant comprising plant tissue, wherein the plant tissue, when orally administered to the animal, elicits an antigenic response.

91. (Amended)

The transgenic plant of claim 73 comprising plant tissue, wherein at least some of the plant tissue is edible by animals.

98. (Twice Amended)

A transgenic plant having plant tissue[,] wherein said plant tissue is expressing a recombinant protein, said protein expressed at a level such that when said plant tissue is orally administered to the animal, an immune response is elicited against an animal viral antigen.

99. (Amended)

A plant comprising a protein which triggers production of antibodies to hepatitis virus or transmissible gastroenteritis virus protein, said protein being a product produced by the method of: expressing said protein in a transgenic plant, said transgenic plant being in a form chosen from the group consisting of a whole plant, plant part, or a crude plant extract.

100. (Amended)

A plant comprising a protein which triggers production of antibodies to a viral protein, said protein being a product produced by the method of: expressing said protein in a transgenic plant, said transgenic plant being in a form chosen from the group consisting of a whole plant, plant part, or a crude plant extract.

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Expression of Immunogenic Glycoprotein S Polypeptides from Transmissible Gastroenteritis Coronavirus in Transgenic Plants

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The use of transgenic plants as vaccine production systems was described recently. We report on the immunological response elicited by two recombinant versions of the glycoprotein S from the swine-transmissible gastroenteritis coronavirus (TGEV) expressed in transgenic plants. Arabidopsis plants were genetically transformed with cDNAs constructs encoding either the N-terminal domain (amino acid residues 1–750) or the full-length glycoprotein S of TGEV, responsible for the neutralizing antibody induction against the virus, under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. Genomic DNA and mRNA analyses of leaf extracts from transformed plants demonstrated the incorporation of the foreign cDNA into the Arabidopsis genome, as well as their transcription. Expression of recombinant polypeptides were observed in most transgenic plants by ELISA using specific antibodies. Mice immunized with leaf extracts from transgenic plants developed antibodies that reacted specifically with TGEV in ELISA, immunoprecipitated the virus-induced protein, and neutralized the virus infectivity. From these results, we conclude that transgenic plants expressing glycoprotein S polypeptides may possibly be used as a source of recombinant antigen for vaccine production. © 1998 Academic Press

INTRODUCTION

Swine-transmissible gastroenteritis virus (TGEV) is the causative agent of acute diarrhea of newborn piglets that provokes high mortality rates in affected farms. Protective immunity against this disease must be developed in pregnant sows to confer passive protection to the piglets through colostrum and milk. Neutralizing antibodies against the virus are directed mainly to glycoprotein S (Garwes *et al.*, 1978; Jimenez *et al.*, 1986), and relevant epitopes in neutralization have been mapped into the N-terminal domain of this protein (Correa *et al.*, 1988). Four major antigenic sites have been described in glycoprotein S, of which site A is the immunodominant (De Diego *et al.*, 1992; Delmas *et al.*, 1990; Sánchez *et al.*, 1990). Glycoprotein S from TGEV has been expressed using different vectors with tropism that favored antigenic presentation in the mucosal surfaces (Smerdou *et al.*, 1996; Torres *et al.*, 1995). These vaccination approaches promoted systemic and mucosal antibody induction and, in the case of adenovirus vector, conferred protection to suckling piglets (Torres *et al.*, 1996).

The development of genetic transformation technology in plants has made possible the expression of foreign genes in different plant species, making reasonable the idea of using plants as bioreactors to produce recombinant proteins. The concept of vaccine production in transgenic plants was first introduced by Mason *et al.* in 1992. Proteins involved in protective immune response can be produced at a low cost and easily purified from plant extracts for parental inoculation. In addition, oral immunization by edible vaccines produced in transgenic plants could stimulate immune responses at the portal entrance of many pathogens, facilitating the design of large-scale immunization programs. The presence of specific antigens into plants, even at low levels, can raise by the oral route immune reactions comparable to those raised by conventional vaccines (Haq *et al.*, 1995; Mason *et al.*, 1996).

Hepatitis B surface antigen (Thanavala *et al.*, 1995), *Escherichia coli* heat-labile enterotoxin (LT-B) antigen (Haq *et al.*, 1995; Tacket *et al.*, 1998), Norwalk virus capsid protein (Mason *et al.*, 1996), VP1 antigen from foot and mouth disease virus (Carrillo *et al.*, 1998), and cholere toxin B subunit (Arakawa *et al.*, 1998) are the vaccine antigens expressed in transgenic plants and tested for the immune response elicited in immunized animals. Additionally, rabies virus glycoprotein was expressed in transgenic tomatoes, but the immune response induced by administration of these plants to animals was not tested (McGarvey *et al.*, 1995). In the present study, we investigated the feasibility of expressing the glycoprotein S from TGEV in transgenic plants, as well as the antigenicity and immunogenicity of the plant-derived protein. The S protein is an excellent model for developing oral

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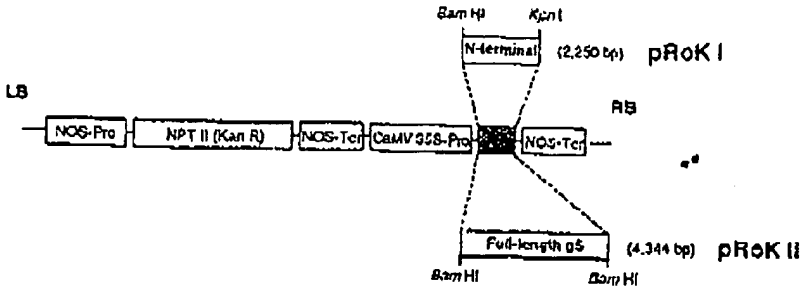

FIG. 1. Schematic structure of the binary plasmids pRoK I and II used for *Agrobacterium*-mediated plant transformation. The DNA sequences encoding for the full-length or N-terminal domain of glycoprotein S from TGEV are cloned downstream of the CaMV 35S promoter in recombinant pRoK plasmids, followed by the nopaline synthase (NOS) terminator. These plasmids contain the left (LB) and right (RB) borders of transferred DNA that demarcate the sequences that are incorporated into the plant genome.

vaccines against enteric pathogens of mammals because of its immunogenicity and resistance to degradation in the gut.

RESULTS

Plasmid construction and selection of transgenic plants

The binary pRoK I and pRoK II recombinant plasmids (Fig. 1), carrying a cDNA coding for the N-terminal region or the full-length glycoprotein S respectively, were obtained by subcloning the corresponding sequences from previously obtained constructs. Recombinant pRoK plasmids allow selection of transformants on media containing kanamycin and stable integration into nuclear chromosomal DNA from the plant. pRoK uses the cauliflower mosaic virus 35S (CaMV 35S) promoter for nominally constitutive transcription of the cloned genes.

Plant transformation with pRoK I and II was carried out as described in Materials and Methods by *Agrobacterium tumefaciens*-mediated transformation. The transgenic plants resistant to the selective medium appeared similar in morphology to the nontransgenic arabidopsis plants. More than 20 different lines of transformants containing each construct were obtained and self-pollinated to obtain F2 lines. All lines were positive when screened for the presence of the recombinant genes by polymerase chain reaction (PCR) analysis (Fig. 2A).

Most plants harboring recombinant genes showed specific transcription of foreign genes by reverse transcription (RT)-PCR analysis (Fig. 2B). To rule out the possibility of amplification of contaminant DNA sequences present in the RNA preparations, we treated the purified RNA with ribonuclease before foreign gene amplification by using *Taq* polymerase. No amplified DNA fragments were detectable under those conditions, assessing the RNA dependence of the reaction (Fig. 2B).

Recombinant protein expression in transgenic plants

The presence of the recombinant polypeptides in the plants harboring and expressing the foreign gen s was investigated in four plants of each construct, selected to be analyzed by ELISA and Western blotting using an anti-TGEV polyclonal serum. Results demonstrated that leaf extracts from all selected plants were positive on ELISA (Fig. 3). However, no specific reaction on Western blotting was detected in any of the plant extracts analyzed (data not shown), probably due to the low levels of recombinant protein expression and to the conformational nature of most of the immunodominant epitopes present in this protein.

From a titration ELISA using different virus dilutions and a monospecific anti-glycoprotein S antibody, we found that ~30–60 μg of soluble leaf protein contains a glycoprotein S antigenic mass equivalent to that contained in 0.02 μg of purified TGEV. The percentage of the total soluble protein corresponding to recombinant glycoprotein S polypeptides accumulated in the leaves of arabidopsis transformants could represent 0.06–0.03% of the total soluble leaf protein.

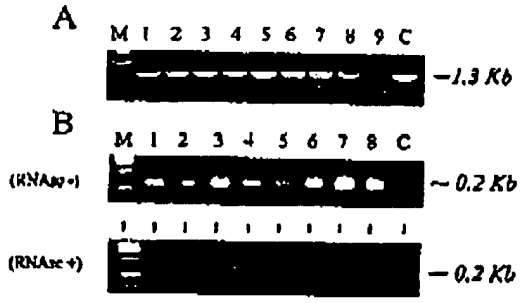

FIG. 2. Characterization of transgenic plants transformed with pRoK I (1–4), pRoK II (5–8), or pRoK 2 (9) plasmids. (A) Presence of the recombinant genes in representative transgenic arabidopsis plants detected by PCR. C, control amplification of the same DNA fragment from pRoK II. (B) Foreign gene transcription in representative transformed plants analyzed by RT-PCR. Samples were treated or not with RNase to assess the DNA specificity of the reactions. C, same analyses in arabidopsis plants transformed with pRoK 2 plasmid.

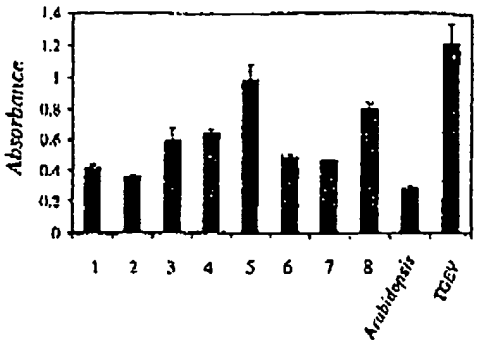

FIG. 3. Detection of N-terminal (1–4) and full-length (5–8) glycoprotein S polypeptides in protein extracts from transgenic plant leaves by ELISA. The figure shows the mean ± SD of the absorbance readings obtained in three independent assays. Positive control is purified virus (TGEV), and negative control is a plant transformed with pRok 2 plasmid (*Arabidopsis*).

Antibody induction by plant-derived recombinant proteins

Leaf extracts from transgenic plants expressing the N-terminal (plants 1–4) or full-length glycoprotein S (plants 5–8) were used to immunize mice. A control mouse was immunized with a leaf extract from a plant transformed with pRok2 plasmid. After three immunization doses, the specificity of mice sera was tested by an ELISA using purified TGEV as antigen. Figure 4A shows that all sera reacted with the virus showing, as expected, different titers. A kinetic of antibody induction in an immunized mouse (number 3) was studied by immunoprecipitation of glycoprotein S induced by TGEV in infected ST cells. This mouse serum immunoprecipitated specifically the virus protein after two immunizations (Fig. 4B).

Finally, sera from all immunized mice were tested in a TGEV neutralization assay. Both glycoprotein S polypeptides produced in transgenic plants elicited virus-neutralizing antibodies (neutralization indexes of 2.2–3.6; Fig. 4C). Serum from a nonimmunized mouse (not shown) or from the mouse immunized with the plant transformed with pRok 2 plasmid did not show virus neutralization activity (Fig. 4C).

DISCUSSION

In this report we show that full-length or the globular part (N-terminal domain) of TGEV spike protein (glycoprotein S) expressed in transgenic plants retained the antigenic properties and elicited neutralizing antibodies when used to immunize animals. Expression in eukaryotic hosts is required for antigenic determinants that are dependent on glycosylation. Of the three major antigenic sites defined on glycoprotein S involved in the induction of TGEV-neutralizing antibodies, sites A and B are complex, conformational, and glycosylation dependent. Site D can be represented by synthetic peptides, although glycosylation has a minor effect on its conformation (Gebauer *et al.*, 1991). Several genetically engineered vaccines using prokaryotic vectors have failed against TGEV. Glycoprotein S expressed at high levels in *Escherichia coli* and used to inoculate animals did not induce neutralizing antibodies or confer protection *in vivo* (Hu *et al.*, 1987).

Plant cells present differences in protein glycosylation with respect to animal cells that could determine the lose of antigenic determinants in antigens expressed in transgenic plants. Glycosylation in plants may differ in the extent of glycosylation, processing, or both of N-linked oligosaccharide side chains (Faye *et al.*, 1993). Furthermore, the complex glycans of plants are often smaller than those of animals, in part due to the absence of sialic acid (Faye *et al.*, 1993). The only precedent of a glycoprotein expressed in plants for vaccine development is the glycoprotein G of rabies virus (McGarvey *et al.*, 1995). This protein expressed in tomato plants showed a molecular mass ~4 and ~6 kDa less than that obtained from virus-infected cells but still larger than the protein size predicted for the unglycosylated polypeptide chain (McGarvey *et al.*, 1995). The molecular mass of glycoprotein S expressed in *Arabidopsis thaliana* could not be determined because we were not able to detect the recombinant protein on Western blotting. However, antigenic determinants with strong dependence of glycosylation seem to be preserved because the plant-derived antigens induced neutralizing antibodies in immunized animals, indicating that critical antigenic sites are at least in part correctly glycosylated in plants.

This work demonstrates the feasibility of expressing glycoprotein S polypeptides in plants. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is random, different levels of protein expression in independent transformants are expected. We obtained expression levels similar to that described with equivalent constructs expressing hepatitis B surface antigen or rabies virus glycoprotein (Mason *et al.*, 1992; McGarvey *et al.*, 1995). More recently, expression levels of Norwalk virus capsic protein in tobacco have been shown to be higher than the above mentioned antigens (up to 0.23% of total soluble protein; Mason *et al.*, 1996). We have not found significant differences in foreign antigen plant expression between the two forms of glycoprotein S studied. The use of different promoters, the use of plant-derived leader sequences and signal peptides, and mainly the modification of the codon usage of this protein could improve expression levels in plants.

The demonstration that many proteins from pathogens, including some expressed in transgenic plants (Haq *et al.*, 1995; Mason *et al.*, 1996), are immunogenic when administered orally, encourages the study of other antigens expressed in plants to develop edible vaccines.

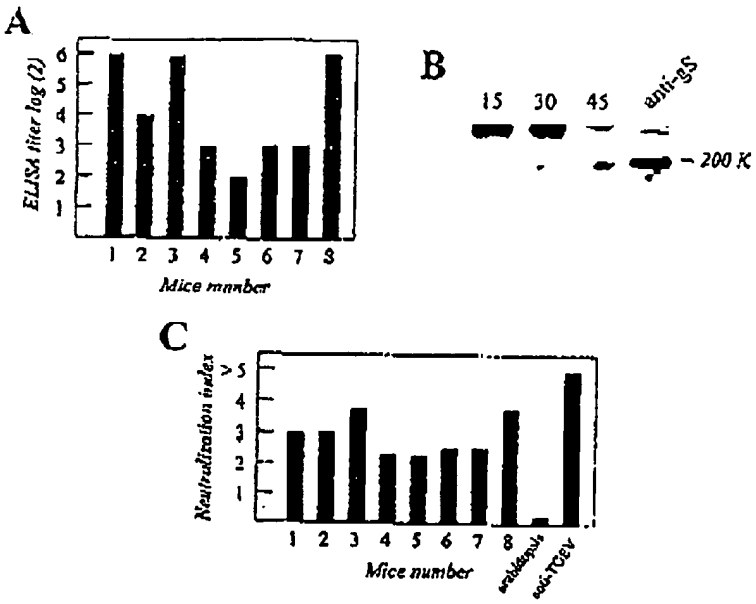

FIG. 4. Antibody responses to the plant-derived glycoprotein S polypeptides in parenterally inoculated mice. (A) ELISA titers of sera from mice inoculated with plant extracts expressing the N-terminal (mice 1–4) or the full-length glycoprotein S (mice 5–8). Titers are referred to the ELISA values obtained with the serum from a mouse immunized with a pRoK 2 transformed plant extract. (B) Kinetic of antibody induction against glycoprotein S in a mouse (mouse 3) after one, two, and three immunization doses (15, 30, and 45 days postinoculation, respectively) analyzed by immunoprecipitation of the glycoprotein S induced by TGEV in infected cells. An anti-glycoprotein S (anti-gS) serum was used as immunoprecipitation control. (C) Neutralization indexes of sera from immunized mice with plant extracts expressing the N-terminal (mice 1–4) or full-length glycoprotein S (mice 4–8). The neutralization index is defined as the ratio between the log of virus titer in the presence of a control mouse serum and sera from mice immunized with transgenic plants expressing the antigens (1–8) or transformed with pRoK 2 (*Arabidopsis*). A rabbit anti-TGEV serum (anti-TGEV) showing high neutralization titer was also used as positive control. ELISA and neutralization index values are the mean of three independent experiments.

Glycoprotein S from TGEV is an interesting model because this protein is resistant, at least when incorporated into the viral particle, to gut degradation. In addition, the protective immune responses against TGEV have to be stimulated at the mucosal surfaces to induce secretory and lactogenic immunity (De Diego *et al.*, 1992, 1993; Saif and Bohl, 1979; Wesley *et al.*, 1988). Once we have determined the feasibility of expressing immunological active polypeptides from TEGV glycoprotein S in plants, studies on the immune response of plant-derived glycoprotein S polypeptides in pigs are necessary.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (Heynh, ecotype Columbia) were sown in pots containing a mixture of universal substrate and vermiculite (3:1). To synchronize germination, pots were placed at 4°C for 48 h in darkness and then transferred to a growth chamber at 20°C with a 16-h photoperiod. Irrigation was carried out with distilled water and, occasionally, with a mineral nutrient solution (Haughn *et al.*, 1986).

Production of transgenic *Arabidopsis*

A 2250-pb cDNA fragment (nucleotides 1–2250; fragment I) and a 4344-pb cDNA fragment (nucleotides 1–4344; fragment II) encoding for the N-terminal and full-length glycoprotein S from TGEV Purdue strain, respectively, were amplified by RT-PCR from viral RNA and cloned into pBacPAK9 plasmid (Clontech). The RT primers used were 5′-CCCAACTATGGTACCATCAAT AACAGC-3′ (complementary primer to nucleotides 2225–2250) and 5′-CGCGGGATCCTTAATGGACGTG-CACTTTTTC-3′ (complementary primer to nucleotides 4313–4344). Then, the cDNA was synthesized by using the primer 5′-GCGCGGATCCATGAAAAACTAT I TGT-GG-3′. Subsequently, DNA fragments I and II were subcloned in the binary pRoK2 plasmid (Baulcombe *et al.*, 1985) under the control of the CaMW 35S promoter, yielding the recombinant plasmids pRoK I and pRoK II, respectively (Fig. 1).

Plasmids pRoK I and pRoK II were used for arabidopsis plant transformation as described elsewhere (Bechtold *et al.*, 1993) with slight modifications. *A. tumefaciens* (C58C1 strain) containing pRoK I or pRoK II plasmids was grown in 600 ml of LB medium con-

taining 50 μg/ml kanamycin until an OD_{600} value of 2 was reached. After centrifugation, bacteria were resuspended in 200 ml of 2.35 g/l Murashige and Skoog medium containing 10 g/l 6-benzilaminopurine and 5% sucrose. The 6-7-week-old plants were immersed in the *A. tumefaciens* suspension by inversion of the pots, and vacuum infiltration was performed in a vacuum chamber at 50 mb for 15 min. Infiltrated plants were rinsed with water and placed in the greenhouse until attaining maturity. Transgenic T1 seeds were selected by germination in Petri dishes containing GM (4.7 g/l Murashige and Skoog, 1% sucrose, 0.5 g/l 2-(*N*-morpholino)ethanesulfonic acid (MES), 8 g/l agar, pH 5.7) and 50 μg/ml kanamycin. Two-week-old transgenic plants were transplanted into soil and allowed to attain maturity. The plants were self-pollinated to obtain T2 plants and used for further analysis.

PCR and RT-PCR analyses

The presence of the foreign cDNA sequences in generated transgenic arabidopsis was detected by PCR. Plant extracts were prepared by macerating leaves (~10 mg) with pestle and mortar in 300 μl of a buffer containing 200 mM Tris–HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. The resulting extract was mixed with 150 μl of 3 M CH_3COONa, pH 5.2, and incubated for 10 min at −20°C. Then, samples were centrifuged, and the DNA contained in the supernatant was precipitated and resuspended in 30 μl of TE buffer. PCR was performed on 0.5 μg of DNA with a pair of primers that specifically amplify a 1389-bp fragment of the glycoprotein S gene (sense primer, 5′-GCGCGGATCCATGAAAAACTATTTGTGG-3′; antisense primer, 5′-GCGCGGTACCCGATGTGAAGCTATTG-3′).

Glycoprotein S mRNA in transgenic plants was analyzed by RT-PCR. Total RNA from the leaves of transformed plants was isolated using the Fast RNA kit (BIO 101) according to the manufacturer's instructions. RNA was treated with 10 units of DNase–RNase free (RQ1; Promega) during 15 min at 37°C. Then, 1 μg of total RNA was diluted in 4 μl of RT buffer [250 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 375 mM KCl, and 10 mM dithiothreitol (DTT)] containing 0.5 mM concentration of each dNTP (Pharmacia), 10 units of RNasin (RNase inhibitor, human placenta; Boehringer-Mannheim), 200 units of reverse transcriptase (Moloney murine leukemia virus RT; GIBCO BRL), and 100 pM concentration of the antisense primer (5′-GCGCGGTACCAAACCAAGGTTGTACAG-3′) in a final volume of 20 μl. The mixture was incubated for 1 h at 37°C. Denaturation of RNA–cDNA hybrids and inactivation of the reverse transcriptase were done by boiling the reaction for 5 min. To the RT mixture, we added 50 pM concentration of the above mentioned sense primer and 5 units of *Taq* DNA polymerase (GIBCO BRL). This reaction specifically amplifies a 197-bp fragment of the glycoprotein S gene. Treatments of purified RNA samples with RNase (Promega) were carried out with 10 units of the enzyme for 15 min at 20°C.

Detection of glycoprotein S in transgenic plants

Proteins from leaves were obtained by homogenization of leaves in a blender with liquid nitrogen, and the resulting powder was resuspended in buffer (0.3 g of fresh wt/ml) containing 10 mM MES, pH 6, 10 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. The extract was filtered and centrifuged 10 min at 12,000 *g*, and the resulting supernatant was used for glycoprotein S polypeptides expression analyses.

ELISA plates were coated with 100 μl (10 μg/ml of PBS) of a mixture of two monoclonal antibodies, 6AC3 and 6DH8 (kindly provided by Dr. L. Enjuanes, Centro Nacional de Biotechnologia, CSIC, Spain), recognizing the antigenic sites A and D of the glycoprotein S, respectively (Correa *et al.*, 1988). Antibodies were incubated for 12 h at 4°C, and then plates were washed and blocked 1 h at 37°C with 5% fetal bovine serum in PBS containing 0.05% Tween 20. After washing the plates, leaf proteins from transgenic plants (15 μg of total soluble protein per well, diluted in 200 μl of PBS, pH 7), containing full-length or the N-terminal domain of glycoprotein S, were added to react with the previously adsorbed antibodies in the microtiter ELISA plates during 12 h at 4°C. Plates were then washed six times with 0.05% Tween 20 in PBS, and 100 μl of rabbit anti-S protein, obtained after three immunization doses with the baculovirus-expressed N-terminal fragment of glycoprotein S and diluted at 1:100 in PBS containing 0.05% Tween 20, was added per well and left to react for 1 h at 37°C. Plates were washed again six times with PBS–Tween 20 buffer, and immunocomplexes were incubated with Protein A-peroxidase (Sigma) diluted 1:1000 in PBS–Tween 20 for 1 h at 37°C. Finally, plates were washed again, and 200 μl of a freshly prepared solution of *o*-phenylenediamine dihydrochloride (Sigma) and H_2O_2 was added. Reactions were stopped with 2 N H_2SO_4, and the absorbance was measured at 492 nm.

Induction of anti-glycoprotein S antibodies

BALB/c mice (one per arabidopsis plant) were immunized intramuscularly on days 0, 15, and 30 with leaf extract in PBS (40 μg of total protein per animal per injection) in complete Freund's adjuvant for the first inoculation and in incomplete adjuvant for the others.

Mice sera were evaluated for anti-glycoprotein S-specific antibodies by ELISA using purified TGEV as antigen. Coated ELISA plates with 100 μl of PBS, pH 7.4, containing 0.2 μg of virus were blocked as described above with

5% fetal bovine serum, and after washing of the plates six times, sera diluted 1:10 in PBS–Tween 20 were added (100 μl per well) and incubated for 1 h at 37°C. Then, plates were washed again to remove unbound antibodies, and goat anti-mouse antibodies (1:500) were added to reveal immunocomplexes. After being washed and developed with o-phenylenediamine dihydrochloride substrate as described above, reaction was stopped with 2 N H_2SO_4, and plates were read at 492 nm.

Immunoprecipitation of glycoprotein S by sera from a mouse after different immunization doses was carried out essentially as previously described for mouse antibodies (Bullido *et al.*, 1996). Briefly, ST cells infected with TGEV (m.o.i. 5) were incubated for 14 h, pulse labeled for 1 h with 200 μCi/ml of ^{35}S-methionine (800 Ci/mmol; Amersham International, Amersham, England)/ml, and lysed with lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.4, 1 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride). The lysate (10^6 cpm) was incubated with a control mouse serum (15 μl) for 1 h and precleared with a 25% (v/v) suspension of Protein G–Sepharose (Pharmacia, Sweden) in lysis buffer. The precleared ^{35}S-labeled cell extract was incubated with mice sera (15 μl) for 2 h at 4°C, and immunocomplexes were incubated with 25% suspension of Protein G–Sepharose for 1 h with gentle mixing. Beads were washed three times with lysis buffer and boiled in SDS–electrophoresis buffer. The antigen–antibody complexes were analyzed in 7.5% SDS–PAGE.

A plaque reduction assay with sera from immunized mice was performed as described previously (Jiménez *et al.*, 1986). The neutralization index of each serum was expressed as the $\log_{10}$ of the ratio of the pfu/ml of virus obtained using a normal serum and that observed in the presence of a given anti-glycoprotein S mouse serum.

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Immunology

Expression of hepatitis B surface antigen in transgenic plants

(oral vaccine/foreign genes/plants)

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ABSTRACT **Tobacco plants were genetically transformed with the gene encoding hepatitis B surface antigen (HBsAg) linked to a nominally constitutive promoter. Enzyme-linked immunoassays using a monoclonal antibody directed against human serum-derived HBsAg revealed the presence of HBsAg in extracts of transformed leaves at levels that correlated with mRNA abundance. This suggests that there were no major inherent limitations of transcription or translation of this foreign gene in plants. Recombinant HBsAg was purified from transgenic plants by immunoaffinity chromatography and examined by electron microscopy. Spherical particles with an average diameter of 22 nm were observed in negatively stained preparations. Sedimentation of transgenic plant extracts in sucrose and cesium chloride density gradients showed that the recombinant HBsAg and human serum-derived HBsAg had similar physical properties. Because the HBsAg produced in transgenic plants is antigenically and physically similar to the HBsAg particles derived from human serum and recombinant yeast, which are used as vaccines, we conclude that transgenic plants hold promise as low-cost vaccine production systems.**

Hepatitis B virus infection is one of the most widespread viral infections of humans and causes acute and chronic hepatitis and hepatocellular carcinoma (1). The infectious viral particle (Dane particle) is a 43-nm double-shelled sphere that consists of a core containing the 3.2-kilobase (kb) DNA genome bound to the core protein, surrounded by the viral envelope containing phospholipids and the major surface antigen [hepatitis B surface antigen (HBsAg)] (2). In addition to Dane particles, the serum of infected individuals also contains 22-nm subviral particles in great excess over virions. These noninfectious particles contain the elements of the viral envelope, including the major 24-kDa peptide that occurs in glycosylated and unglycosylated forms (2).

Because the host range of hepatitis B virus is limited to humans and chimpanzees, and since the virus cannot be propagated in cell culture, HBsAg for use in vaccines was purified from the serum of infected individuals until a recombinant form (rHBsAg) was produced in yeast (3). The immunogenic yeast-derived rHBsAg occurs in the form of spherical particles with an average diameter of 17 nm. Integration of the peptides into the phospholipid-containing particles greatly enhances their immunogenic properties (4). Subsequent work showed that the peptides present in the yeast-derived particles were much less extensively disulfide-linked than in the human material but that such linking could be induced *in vitro* (5).

Intramuscular injection of serum-derived HBsAg or yeast-derived rHBsAg in healthy individuals results in effective immunization and protection from viral infection (6, 7). In many areas of the developing world, however, the expense of immunization programs for large segments of the population is prohibitive. This has led us to attempt the expression of rHBsAg in plants with the hope of developing a less expensive production system. Further, we hope to find a way to present the rHBsAg in edible plant tissues in a form that would be useful as an oral vaccine. In this paper we describe the transformation of tobacco with the gene encoding HBsAg and its expression in leaf tissue in the form of an antigenic spherical particle with an average diameter of 22 nm. This plant-derived rHBsAg is directly analogous to the rHBsAg from yeast that is now used for commercial vaccines. We view this as a successful first step in a long-term project dedicated to developing technologies for low cost "edible vaccines" for the developing world.

MATERIALS AND METHODS

Construction of Plasmids for Plant Transformation. The HBsAg coding region on the *Pst* I/*Hin*dIII fragment from pMT-SA (kindly provided by Li-he Guo, Chinese Academy of Sciences) was subcloned into pBluescript KS (Stratagene) to form pKS-HBS. The HBsAg gene in pKS-HBS was opened 116 base pairs (bp) 3′ to the termination codon with *Bst*BI and the resulting ends were blunted by filling with Klenow enzyme and dCTP/dGTP. The entire coding region was then excised 16 bp upstream of the *Pst* I site with *Bam*HI. pBI121 (ref. 8; obtained from Clontech) was digested with *Sac* I and the ends were blunted with mung bean nuclease. The GUS coding region was then released with *Bam*HI and the vector was isolated. The HBsAg coding fragment was ligated into the GUS-less pBI121 to yield pHB101 (Fig. 1), where its expression is driven by the cauliflower mosaic virus (CaMV) promoter derived from pBI121.

The CaMV 35S promoter with duplicated enhancer linked to the tobacco etch virus (TEV) 5′ nontranslated leader sequence, which acts as a translational enhancer (9), was excised from pRTL2-GUS (10) as follows. pRTL2-GUS was digested with *Nco* I and the ends were blunted with mung bean nuclease. The promoter-leader fragment was then released by digestion with *Hin*dIII. pHB101 was digested with *Hin*dIII and *Sma* I to release the 35S promoter fragment, and the vector was purified. The promoter-leader fragment was then ligated into the *Hin*dIII/*Sma* I-digested pHB101 to yield pHB102 (Fig. 1). The HBsAg coding region lies upstream of the nopaline synthase terminator in both constructs. The plasmids contain the left and right border regions, which denote the limits of the DNA that is integrated into the plant genomic DNA via *Agrobacterium tumefaciens*-mediated transformation, as well as the neomycin phosphotransferase gene, which allows selection with kanamycin.

Plant Transformation. *Agrobacterium* strain LBA4404 cells were transformed by the direct method (11) with the plasmids prepared from *Escherichia coli* clones, and the

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Abbreviations: CaMV, cauliflower mosaic virus; HBsAg, hepatitis B surface antigen; rHBsAg, recombinant HBsAg; TEV, tobacco etch virus.

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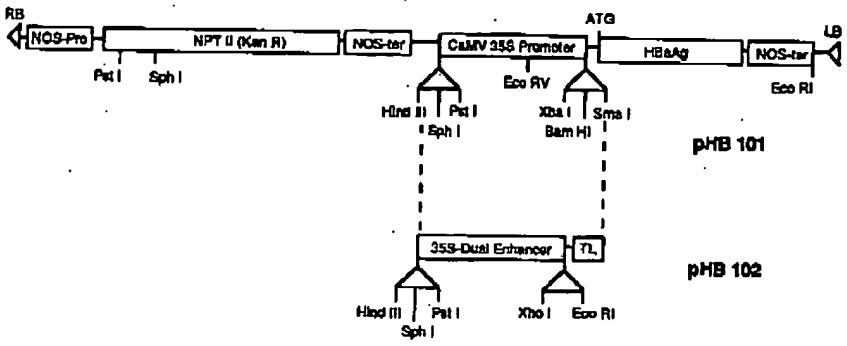

FIG. 1. Structure of plasmids pHB101 and pHB102. The constructs carry the left and right borders (LB, RB) of the transferred DNA that demarcates the sequences that are incorporated into the plant genomic DNA via *Agrobacterium*-mediated transformation. The HBsAg coding region lies downstream of the CaMV 35S promoter in pHB101 and is followed by the nopaline synthase (NOS) terminator. In pHB102, the 35S promoter is replaced by a modified CaMV 35S promoter containing a duplicated transcriptional enhancer region, linked to the TEV 5′ nontranslated leader (TL). Restriction endonuclease cleavage sites are indicated. Blunt-ended ligation has removed the *Nco* I site at the 3′ end of the leader sequence.

structure of the plasmids was verified by restriction digestion. Tobacco (*Nicotiana tabacum* cv. Samsun) was transformed by cocultivating leaf discs (12) with *Agrobacterium* strains transformed with pHB101 or pHB102. Shoots were generated from transformed callus selected on medium containing 0.2 mg of kanamycin per ml and 0.2 mg of cefotaxime per ml. Shoots were rooted in medium containing 0.1 mg of kanamycin per ml, transplanted to soil, and watered with one-half strength Hoagland medium.

Analysis of RNA from Transformed Tobacco. Total RNA from leaves of plants transformed with pHB101 was isolated as described (13). The RNA was denatured with formaldehyde, fractionated on 1% agarose gels (5 μg per lane), blotted to nylon, and probed with ^{32}P-labeled random-primed DNA using a 700-bp *Bam*HI/*Acc* I fragment from pKS-HBS that includes most of the coding region for HBsAg. Blots were hybridized at 68°C in 0.25 M sodium phosphate, pH 7.0/1 mM EDTA/7% SDS, washed with 40 mM sodium phosphate, pH 7.0/5% SDS at 68°C, and exposed to X-Omat AR film for 4 hr.

Analysis of Protein from Transformed Tobacco. Protein was extracted from leaf tissues by homogenization with a Ten-Broek ground glass homogenizer (clearance, 0.15 mm) in 5 volumes of buffer containing 20 mM sodium phosphate (pH 7.0), 0.15 M NaCl, 20 mM sodium ascorbate, 0.1% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride at 4°C. The homogenate was centrifuged at 1000 × *g* for 5 min, and the supernatant was centrifuged at 27,000 × *g* for 15 min. The 27,000 × *g* supernatant was centrifuged at 100,000 × *g* for 1 hr, and the pellet was resuspended in extraction buffer. Protein in the different fractions was measured by the Coomassie dye-binding assay (Bio-Rad). HBsAg was assayed with the Auszyme monoclonal kit (Abbott), using the positive control (HBsAg derived from human serum) as a standard. The positive control was diluted to give HBsAg levels of 0.09–1.8 ng per assay, and the absorbance at 492 nm after color development gave a linear relationship in this range.

Immunoaffinity Purification of HBsAg from Transgenic Tobacco. Monoclonal antibody against HBsAg (clone ZMHB1) was obtained from Zymed Laboratories. The immunogen source for this antibody is human serum. The antibody was bound to Affi-Gel Hz hydrazide gel (Bio-Rad) according to the instructions supplied with the kit. Soluble material that was resuspended from the 100,000 × *g* pellet was made to 0.5 M NaCl and mixed with the immobilized antibody-gel by end-over-end mixing for 16 hr at 4°C. The gel was washed with 10 volumes of 10 mM sodium phosphate, pH 7.0/0.5 M NaCl, and 10 volumes of 10 mM sodium phosphate, pH 7.0/0.15 M NaCl, and bound HBsAg was eluted with 0.2 M glycine (pH 2.5). The eluate was immediately neutralized with Tris base, and particles were pelleted at 109,000 × *g* for 1.5 hr at 5°C. The pelleted material was negatively stained with phosphotungstic acid and visualized with transmission electron microscopy using a Phillips CM10 microscope.

Sucrose and CsCl Gradient Analysis of HBsAg from Transgenic Tobacco. Extracts of leaf tissues were made as described above and 0.5 ml of the 27,000 × *g* supernatants was layered on linear 11-ml 5–30% sucrose gradients made in 10 mM sodium phosphate, pH 7.0/0.15 M NaCl or discontinuous 12-ml CsCl gradients (1.1–1.4 g/ml) made in 10 mM sodium phosphate at pH 7.0 (3 ml each of 1.1, 1.2, 1.3, and 1.4 g of CsCl per ml). Positive control HBsAg from the Auszyme kit was also layered on separate gradients. The sucrose gradients were centrifuged in a Beckman SW41Ti

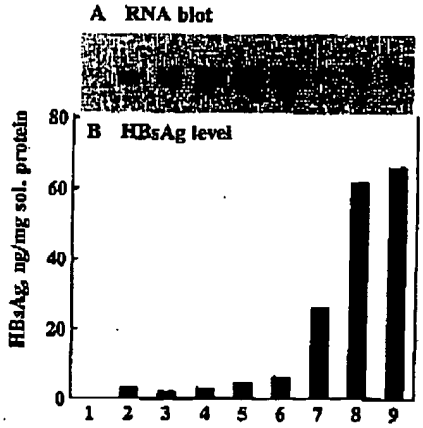

FIG. 2. HBsAg mRNA and protein levels in transgenic tobacco plants. (*A*) Total RNA from wild-type untransformed or independent transgenic tobacco lines carrying either the pHB101 or the pHB102 construct was hybridized with a probe specific for the HBsAg coding region. (*B*) Protein extracts from the same leaves were tested for HBsAg with the Auszyme monoclonal kit (Abbott), and HBsAg levels were quantified using a standard curve of human serum-derived HBsAg. Numbers: 1, wild-type control plant; 2–6, independent transformants harboring the construct in pHB101; 7–9, independent transformants harboring the construct in pHB102.

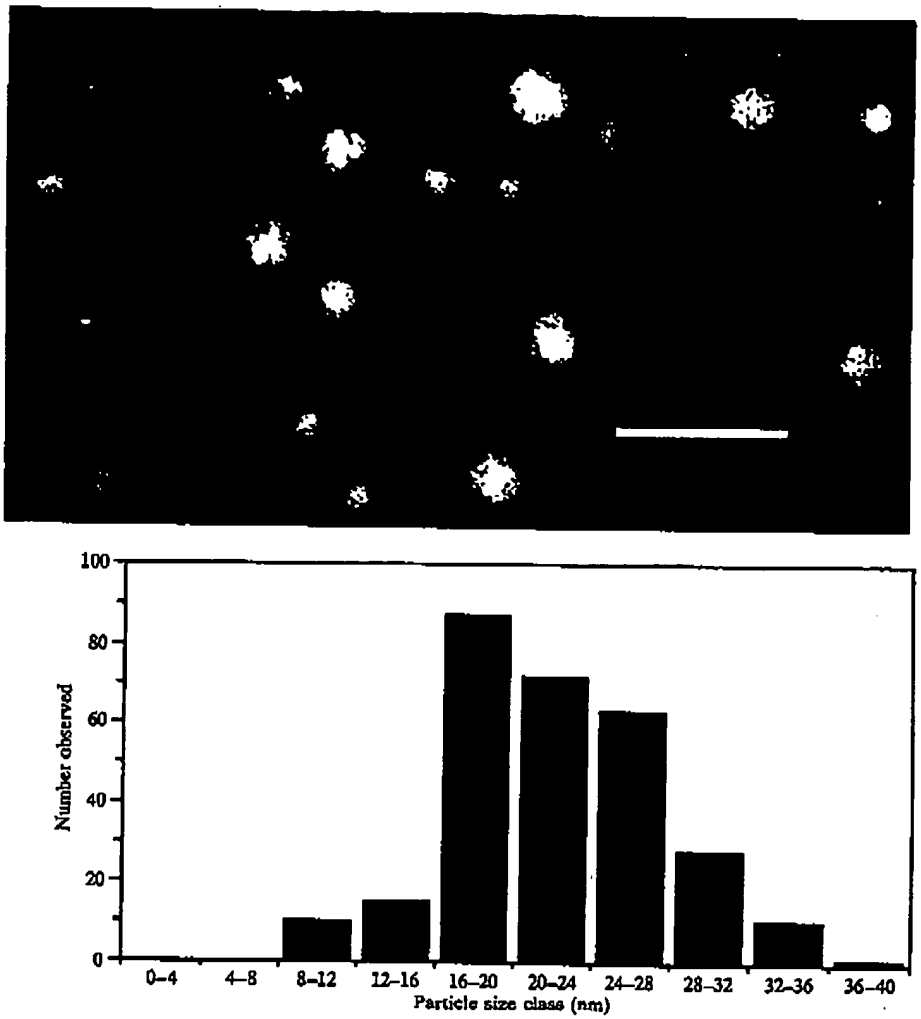

FIG. 3. (*Upper*) Electron micrograph of immunoaffinity purified rHBsAg. HBsAg from a transgenic tobacco plant harboring construct pHB102 was purified by immunoaffinity chromatography, negatively stained with phosphotungstic acid, and visualized by transmission electron microscopy. (Bar = 100 nm.) (*Lower*) Histogram generated by measuring the diameters of the particles observed in the representative field.

rotor at 33,000 rpm for 5 hr at 5°C and fractionated into 1-ml fractions while monitoring the A_{280}. The CsCl gradients were centrifuged in a Beckman SW40Ti rotor at 30,000 rpm for 25 hr at 5°C and fractionated into 1.0-ml fractions. HBsAg in the gradient fractions was assayed using the Auszyme kit as described above. The density of gradient fractions was measured by weighing aliquots with an analytical balance.

RESULTS AND DISCUSSION

The plasmid HB101 (Fig. 1) was constructed by inserting the coding region for HBsAg from pMT-SA between the *Bam*HI and *Sac* I sites in the plant transformation vector pBI121 after removal of the GUS coding region. In this construct the expression of HBsAg is driven by the CaMV 35S promoter. A modification of pHB101 was made by inserting the CaMV 35S promoter with dual transcriptional enhancer linked to the TEV 5′ nontranslated leader (10) in the place of the original 35S promoter to form pHB102 (Fig. 1). The TEV leader acts as a translational enhancer to increase the amount of protein made using a given amount of template mRNA (9).

The plasmids were used to transform tobacco by the leaf disc method using *Agrobacterium*, and regenerated kanamycin-resistant transformants were analyzed by hybridizing RNA samples with a labeled probe encompassing the coding region of the HBsAg gene. Fig. 2*A* shows the results of an experiment where selected transformants harboring either the pHB101 or the pHB102 construct and a wild-type control were probed. The signals were variable between transformants, as expected due to effects of position of insertion into the genomic DNA and differing copy number. The transcripts from the pHB101 transformants (Fig. 2*A*, lanes 2–6) were ≈1.2 kb in length by comparison with RNA standards (H.S.M., unpublished data), which is consistent with the expected size. The pHB102 transcripts were slightly larger (Fig. 2*A*, lanes 7–9), owing to the 5′ addition of the TEV leader sequence. The nontransformed control leaf RNA (Fig. 2*A*, lane 1) showed no detectable signal at this stringency of hybridization. Thus, mRNA that hybridizes specifically with HBsAg probe was present in the leaves of selected transformants, and there is no inherent transcriptional limitation to the expression of HBsAg in tobacco leaves.

Using the HBsAg assay kit, we tested leaf extracts for the presence of material that reacts specifically with monoclonal antibody to serum-derived HBsAg. Fairly low levels were observed for the pHB101 transformants, ranging from 2 to 6 ng/mg of soluble protein (Fig. 2*B*, nos. 2–6). The pHB102 transformants showed substantially greater levels of HBsAg, ranging up to 66 ng/mg of soluble protein (Fig. 2*B*, nos. 7–9). The reaction was specific because wild-type tobacco showed no detectable HBsAg (Fig. 2*B*, no. 1). The levels of HBsAg observed in the individual transformants were roughly proportional to the levels of specific mRNA encoding HBsAg for a given construct. The pHB102 transformants, containing the 5′ TEV leader, showed much higher accumulations of HBsAg for a given amount of mRNA than did the pHB101 transformants (Fig. 2). The translational enhancement observed in mRNAs carrying the TEV leader appears to involve a cap-independent competition for translation initiation factors (9).

HBsAg from human serum occurs as ≈22-nm spherical particles, consisting of protein embedded in a phospholipid bilayer. Since rHBsAg from plasmid-transformed yeast also occurs as particles of a similar size class, we sought evidence that the recombinant material in tobacco is present as particles. We observed that 95% of the HBsAg in the 27,000 × *g* supernatants of transgenic tobacco leaf extracts pelleted at 200,000 × *g* for 30 min (H.S.M., unpublished data), suggesting a particle form. We purified HBsAg by immunoaffinity chromatography using a monoclonal antibody raised against human serum-derived HBsAg. Inspection of this material by negative staining and transmission electron microscopy revealed the presence of particles ranging in diameter between 10 and 40 nm (Fig. 3). Most of the particles were between 16 and 28 nm (Fig. 3 *Lower*); the average diameter was 22 nm. These are very similar to the particles observed in human serum (2), although we observed no rods. The rHBsAg particles from yeast occur in a range of sizes with a mean of 17 nm (3). We conclude that the rHBsAg made in transgenic tobacco retains the capacity for self-association and thus has the physical properties of human serum-derived HBsAg and rHBsAg from yeast, both of which are highly immunogenic in the particle form.

We obtained further evidence of particle behavior from sedimentation and buoyant density studies of transgenic tobacco leaf extracts. Fig. 4 shows a sucrose gradient profile of HBsAg activity from transgenic tobacco harboring the construct in pHB102. The plant-derived HBsAg sedimented with a peak near the 60S ribosomal subunit, and the serum-derived material sedimented in a somewhat sharper peak just slightly slower. These data are consistent with the finding that human HBsAg sediments at 55 S (14). The observation that the plant material sedimented slightly faster and with a broader peak than the human HBsAg is also consistent with the larger mean size of the plant particles and wider range of sizes (Fig. 3). The buoyant density of the rHBsAg from transgenic tobacco in CsCl was found to be ≈1.16 g/ml (Fig. 5), whereas the human particle showed a density of about 1.20 g/ml. Thus, the rHBsAg from transgenic tobacco exhibits sedimentation and density properties that are very similar to the subviral particles obtained from human serum. Importantly, HBsAg in the particle form is found to be much more immunogenic than that in the form of the peptide alone (4).

The subcellular localization of the HBsAg in our transgenic plants has not been characterized. Particles are observed in the lumen of the endoplasmic reticulum in infected liver cells (15) and appear to be secreted by the constitutive secretory pathway. The peptides contain two signal sequences, one N-terminal and one internal, that together determine a transmembrane orientation of the molecules (2). Whether the rHBsAg particles in leaf cells are secreted to the extracellular space or retained within the cytoplasm is a question that needs to be addressed. Although the HBsAg coding region is part of a larger open reading frame in the viral genome (2), the lack of the pre-S peptide does not alter the formation of particles in transfected mammalian cells (16) or yeast (3). Our construct also contains no pre-S sequences and is thus similar to that used in those studies. We were unable to analyze the size of the HBsAg peptides produced in transgenic tobacco because the monoclonal antibodies that we used failed to recognize the SDS-denatured peptides on SDS/PAGE blots. The antibodies did recognize undenatured HBsAg in dot blots of leaf extracts or blots of whole leaves or seedlings, however (H.S.M., unpublished data).

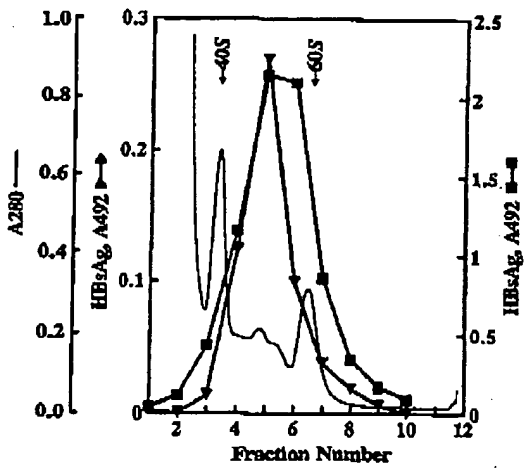

FIG. 4. Sucrose density gradient sedimentation of HBsAg from transgenic tobacco. Soluble fractions from a transformant harboring the pHB102 construct or human serum-derived HBsAg were sedimented in 5–30% sucrose gradients, fractionated, and assayed for HBsAg. The solid curve represents the absorbance profile at 280 nm of the plant extract. The top of the gradient is at the left; positions of the 40S and 60S ribosomal subunits are indicated. ■, HBsAg in transgenic plant extract; ▼, HBsAg in serum-derived material; solid line, absorbance of tobacco leaf extract at 280 nm.

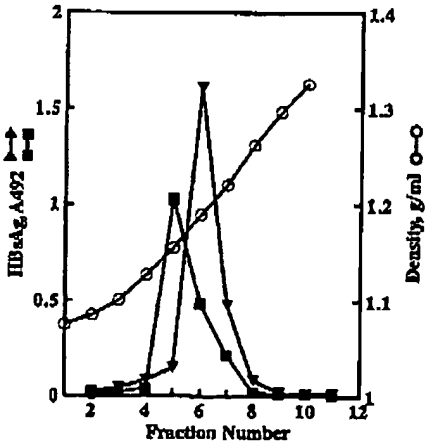

FIG. 5. Buoyant density in CsCl of HBsAg from transgenic tobacco. Supernatant fractions of transgenic tobacco harboring construct pHB102 and human serum-derived HBsAg were banded in a 1.1–1.4 g/ml CsCl gradient. Fractions were assayed for HBsAg activity. ■, HBsAg in plant extract; ▼, HBsAg in serum-derived material; ○, density of gradient fractions.

In conclusion, we have shown that HBsAg can be expressed in plant tissues via stable transformation with foreign DNA. Furthermore, the rHBsAg from transgenic tobacco is recognized specifically by monoclonal antibodies directed against human serum-derived HBsAg and is processed properly after translation so that the antigenic particle form is observed. These studies indicate the feasibility of expression of foreign antigens in plants for possible use as oral vaccines. Presently, the maximal levels of HBsAg we have found in transgenic plants represent ≈0.01% of the soluble leaf protein. This is an inadequate level for the efficient use of plants as production systems for rHBsAg for vaccine use. Further studies must be done to increase the accumulation of HBsAg, such as using other transcriptional regulatory elements to increase mRNA levels. The processing of the HBsAg peptides in plant tissues must also be examined, specifically with regard to glycosylation and intermolecular disulfide bonding. The HBsAg system may be useful for determining the feasibility of targeting foreign antigens to specific subcellular compartments.

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